



Short communication

Non-infectivity of cattle *Streptococcus agalactiae* in Nile tilapia, *Oreochromis niloticus* and channel catfish, *Ictalurus punctatus*Julio C. Garcia^a, Phillip H. Klesius^{a,*}, Joyce J. Evans^b, Craig A. Shoemaker^a^a United States Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Laboratory, 990 Wire Rd, Auburn, AL 36830, United States^b United States Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Laboratory, 118B Lynchburg St, Chestertown, MD 21260, United States

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ABSTRACT

Streptococcus agalactiae is classified as a Lancefield's group B *Streptococcus*. It is one causative bacterium of streptococcosis that is responsible for severe economic losses in wild and cultured fish worldwide. *S. agalactiae* also causes bovine mastitis. No information is available on infectivity of cattle *S. agalactiae* isolates in fish. In the present study, the results showed that 10 cattle isolates from 8 different dairy farms were not infectious in Nile tilapia (*Oreochromis niloticus*) or channel catfish (*Ictalurus punctatus*) at greater than 10⁸ colony forming units per fish. *S. agalactiae* was not re-isolated from the brain or head kidney of the fish at 24 and 48 h post-injection.

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1. Introduction

Streptococcus spp. are considered a diverse group, ranging from commensal organisms which occupy various niches of the human body to pathogens that have the capacity to infect a wide range of hosts (Fishcetti, 2000). *Streptococcus agalactiae* is considered a pathogen responsible for causing infections in chicken, cattle, camels, dogs, bottlenose dolphin, horses, emerald monitors, cats, fish, frogs, hamsters, humans, mice, monkeys and nutria (Wilkinson et al., 1973; Elliott et al., 1990; Wagner and Kaatz, 1997; Evans et al., 2002, 2006a, 2006b; Yildirim et al., 2002a,b; Hetzel et al., 2003; Zappulli et al., 2005). In fish, *Streptococcus* spp. have been reported to cause considerable morbidity and mortality worldwide, estimated losses are \$150 million annually (Klesius et al., 2000).

The pathogenesis of fish *S. agalactiae* isolates is poorly understood. Eldar et al. (1994) reported that *Streptococcus* spp. caused meningoencephalitis in fish. Evans et al. (2002) suggest that *S. agalactiae* isolates from fish are highly virulent and can infect a wide variety of freshwater and saltwater fish. The infectivity of cattle *S. agalactiae* isolates for fish is unknown. It may be possible that cattle could be a source of *S. agalactiae* infection in fish, considering that *S. agalactiae* is present and may be shed from infected dairy cows (Duarte et al., 2004).

In this paper, we determined the infectivity of *S. agalactiae* isolates from cattle in Nile tilapia and channel catfish. We also determined whether isolates of *S. agalactiae* from cattle with mastitis were

phenotypically distinct from *S. agalactiae* isolates from fish. Polymerase chain reaction (PCR) using 16S–23S intergenic rDNA primers (Berridge et al., 2001) was also included to confirm genus and species.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

Thirty six *S. agalactiae* isolates collected from infected mullet, seabream, tilapia, Gulf killifish, and hybrid striped bass in Kuwait, United States, Israel, Honduras and Brazil were used. The isolates obtained from swab samples of the brain, head kidney and eye were cultured on sheep blood agar (SBA) at 32 °C, according to previously published procedures. Identification was based on methods by Evans et al. (2002). Ten *S. agalactiae* isolates were cultured on SBA from the milk of 10 dairy cattle with clinical mastitis from 8 dairy farms and identified as *S. agalactiae* (Department of Pathobiology, Diagnostic Laboratory, College of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin, USA). American Type Culture Collection (ATCC) *S. agalactiae* strains (*n*=2) were included for comparison (Table 1). All isolates were Lancefield grouped using group A, B, C, D, E and G Streptex reagents according to the manufacturer's instructions (Remel, Lenexa, KS, USA).

2.2. Differential characteristics

Differentiation characteristics were determined by capsular serotyping (group B typing antisera Ia, Ib, II and III by the manufacturer's instructions (Denka Seiken, Tokyo, Japan) and fermentation of trehalose and lactose by the manufacturer's instructions API-Strep-

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Table 1

Streptococcus agalactiae isolates, sources and origins from species of marine and fresh water fish and cattle, plus American Type Culture Collection (ATCC) strains of *Streptococcus agalactiae*

Isolate species	Isolate number	Number of isolates	Animal species isolated from	Organ of isolation	Country of origin
<i>Streptococcus agalactiae</i>	ARS-KU-MU ^a	11	Mullet (<i>Liza klunzingeri</i>)	Brain	Kuwait
<i>Streptococcus agalactiae</i>	ARS-KU-SB	6	Seabream (<i>Sparus auratus</i>)	Brain	Kuwait
<i>Streptococcus agalactiae</i>	ARS-BZ-TN ^b	6	Nile tilapia (<i>Oreochromis niloticus</i>)	Brain	Brazil
<i>Streptococcus agalactiae</i>	ARS-BZ-TN ^c	6	Nile tilapia (<i>Oreochromis niloticus</i>)	Brain	Brazil
<i>Streptococcus agalactiae</i>	LADL-US-HSB ^d	1	Hybrid striped bass (<i>Morone saxatilis</i> × <i>M. chrysops</i>)	Brain	USA
<i>Streptococcus agalactiae</i>	LADL-US-TN	1	Nile tilapia (<i>Oreochromis niloticus</i>)	Brain	USA
<i>Streptococcus agalactiae</i>	LADL-HO-TN	1	Nile tilapia (<i>Oreochromis niloticus</i>)	Brain	Honduras
<i>Streptococcus agalactiae</i>	LADL-US-GK	3	Gulf killifish (<i>Fundulus grandis</i>)	Brain	USA
<i>Streptococcus agalactiae</i>	IS-HSB-03 ^e	1	Hybrid striped bass (<i>Morone saxatilis</i> × <i>M. chrysops</i>)	Brain	Israel
<i>Streptococcus agalactiae</i>	US-Cattle ^f	10	Dairy cattle with clinical mastitis from 8 farms	Udder, milk	USA
<i>Streptococcus agalactiae</i>	ATCC-12386 ^g	1	Grouping strain		
<i>Streptococcus agalactiae</i>	ATCC-13813	1	Typing strain		

^a Isolates from Kuwait Bay, Kuwait (Evans et al., 2002).

^b Isolates from Nile tilapia (*Oreochromis niloticus*), reared in cage culture in lakes and ponds in the region of Larvas, Brazil.

^c Isolates from Nile tilapia (*Oreochromis niloticus*) reared in cage culture in lakes and ponds in the region of Parana State, Brazil.

^d Isolates from Dr. John Hawke, Louisiana Veterinary Diagnostic Laboratory, Louisiana State University, Baton Rouge, LA, USA.

^e Isolate from Dr. Dina Zilberg, The Albert Katz Department of Dryland Biotechnologies, Ben-Gurion, University of the Negev, Israel.

^f Isolates from Department of Pathobiology, Diagnostic Laboratory, College of Veterinary Medicine, University of Wisconsin, Madison, WI, USA.

^g Reference strains from American Type Culture Collection (ATCC), Washington, DC, USA.

20 (bioMérieux, Hazelwood, MO, USA)¹. Pigment production (Merritt and Jacobs, 1976) and growth characteristics in fluid medium (Kane et al., 1975) were also determined.

2.3. PCR

Briefly, *S. agalactiae* isolates were incubated in TSB (tryptic soy broth) at 32 °C for 24 h. Samples were added to a sterile 2 mL microcentrifuge tube and centrifuged for 10 min at 5000 ×g. Following centrifugation, the sample supernatant was discarded and bacterial DNA was prepared using the High Pure PCR preparation kit (Roche Diagnostic, Indianapolis, IN, USA) according to their instructions. The eluted DNA was stored at –20 °C (Berridge et al., 2001) until PCR analysis. The method described by Berridge et al. (2001) was used to amplify species-specific 16S–23S rDNA intergenic spacer region of the 48 *S. agalactiae* isolates and type strains (Table 1). Briefly, the PCR reagent mixture contained 1× reaction buffer, 2.5 mM MgCl₂, 0.8 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM each oligonucleotide primer, 0.5 μL of bovine serum albumin, 1.25 units of *Taq* DNA polymerase, 50–100 ng template DNA and sterile water (Berridge et al., 2001). The PCR mixture was cycled 35 times at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min with final extension for 5 min at 72 °C. The annealing temperature was adjusted to 60 °C for the specific *S. agalactiae* primers (forward CCACGATCTAGAAATAGATTG, reverse TGCCAAGG-CATCCACC). The 130 bp PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Samples were considered positive when a clear band of the 130 bp molecular weight product was observed under UV light.

2.4. Experimental infectivity trials – Nile tilapia and channel catfish infected with ten GBS cattle isolates

Non-infected Nile tilapia (*Oreochromis niloticus*; 15.23 ± 1.03 g mean weight) were obtained from USDA-ARS, Aquatic Animal Health Research Laboratory at Auburn, AL. Channel catfish (*Ictalurus punctatus*; 15.0 ± 1.20 g mean weight) were obtained from USDA, ARS, Catfish Genetic Laboratory, Stoneville, MS. The fish were maintained in twenty 57 L aquaria. Mean water temperature was 32 ± 0.6 °C. Fish were exposed to a light and dark period of 12:12 h and were kept in flow-through water (0.5 L/h). Aeration was supplied through air stones, and the fish were fed daily to satiation with Aquamax Grower 400 (Brentwood, MO).

¹ Mention of trade names or commercial products in this publication does not imply endorsement by the U.S. Department of Agriculture.

Ten *S. agalactiae* isolates from cattle (Table 1) were grown in TSB for 24 h at 32 °C. The cattle isolates were obtained from original frozen stock cultures stored at –80 °C. The freshly prepared broth cultures of each isolate were adjusted to an optical density (OD) of 1.0 at 540 nm using a SmartSpec™3000 (Bio Rad, Foster City, CA) and then adjusted to give final concentrations of 1 × 10⁹ and 1 × 10¹⁰ colony forming units (CFU)/mL.

The fish were divided into 6 groups including controls. Two tilapia groups were injected intraperitoneally (IP) with 0.1 mL of 1 × 10⁹ CFU/mL or 1 × 10¹⁰ CFU/mL with each cattle isolate/aquarium. Two catfish groups were injected intraperitoneally (IP) with 0.1 mL with 1 × 10⁹ CFU/mL or 1 × 10¹⁰ CFU/mL with each cattle isolate/aquarium. The fish density was 10 fish/aquarium/group. Two separate aquaria containing 10 control fish each were inoculated with 0.1 mL of TSB. Two fish from each aquarium that were injected with the cattle isolates were removed and their brains and kidneys were cultured on SBA for the re-isolation of the cattle isolates at 24 and 48 h post-injection (Evans et al., 2002).

Two additional separate aquaria served as positive control groups and 10 tilapia each were inoculated with 0.1 mL of a mullet isolate (KU-MU-11-Br) at 1 × 10⁹ CFU/mL or 1 × 10¹⁰ CFU/mL, respectively. This mullet isolate had previously been shown to be virulent for Nile tilapia (Evans et al., 2002), even after repeated passages in TSB. The inoculated fish were then held and observed in 44 individual 57 L aquaria per group for 14 days. Moribund fish were removed and head kidneys and brains were cultured on SBA for *S. agalactiae* isolation (Evans et al., 2002).

3. Results

3.1. Differentiation characteristics between *S. agalactiae* from fish and cattle

Differences in capsular serotypes were found between fish and cattle isolates. Fifty six percent of the fish isolates (mullet and seabream isolates collected from the Kuwait Bay epizootic, Nile tilapia isolates from Brazil and Mississippi, and a hybrid striped bass isolate from Israel) were serotype Ia. Forty four percent of the fish isolates were NT (Nile tilapia isolates from Brazil and Honduras and Gulf killifish and hybrid striped bass isolates from the USA). In contrast, 100% of the cattle isolates (*n* = 10) were non-typable (NT). The type strain, ATCC-13813 was II serotype while ATCC-12386 was non-typable serotype.

The growth patterns of the isolates from mullet, killifish and one half of the hybrid striped bass isolates in fluid medium were

sedimentary while the growth pattern of all seabream and the majority of Nile tilapia isolates were turbid. All of the cattle isolates gave sedimentary growth in fluid medium. Fifty percent of the type strains were turbid.

Pigment production was variable among fish and cattle isolates. The mullet and seabream isolates produced pigment, but not the Nile tilapia, Gulf killifish, hybrid striped bass isolates. None of the cattle isolates produced pigment. All of the type strains produced pigment.

All of the mullet and seabream isolates and one of the Nile tilapia isolates (50%) fermented trehalose. In contrast, 100% of the cattle isolates were negative for trehalose fermentation. All *S. agalactiae* type strains fermented trehalose.

Fermentation of lactose varied between fish and cattle isolates. Fish isolates (7%) were positive. Only one isolate from Larvas, Brazil was positive for lactose. One hundred percent of the cattle GBS isolates and type strains fermented lactose.

3.2. PCR analysis

The 16S–23S rDNA intergenic spacer region primers amplified specific DNA fragments from all fish, cattle isolates and type strains studied (data not shown). These results confirmed that all isolates were *S. agalactiae*.

3.3. Cattle isolates infectivity in fish

Clinical signs and/or mortality did not occur in the cattle isolate challenged catfish, tilapia or TSB control fish. Cattle isolates were not recovered from injected fish at 24 and 48 h post-injection. Tilapia IP injected with the mullet isolate (KU-MU-11-Br) at 1×10^9 CFU/mL and 1×10^{10} CFU/mL had 60 and 90% mortality, respectively. The mullet isolate was recovered from the brain and head kidney at 24 and 48 h post-infection and from the dead tilapia.

4. Discussion

S. agalactiae is the only *Streptococcus* species that has the group B antigen (Facklam, 2002). All of fish, cattle isolates and type strains had group B antigen.

The assumption that reasonable differential characteristics existed between fish cattle *S. agalactiae* isolates was verified. Our results indicate that the capsular serotype, pigment production, D-lactose and D-trehalose fermentation and growth pattern in fluid medium were phenotypic characteristics that were the most useful in distinguishing between fish and cattle isolates. Finch and Martin (1984) demonstrated that cattle and human isolates were separate populations that shared the common group B antigen. In a comparative molecular study between *S. agalactiae* of bovine mastitis and human origins, Bohnsack et al. (2004) indicated that bovine and human *S. agalactiae* isolates were largely unrelated. Numerous studies have evaluated bovine and human *S. agalactiae* isolates phenotypically and variations in results have been noted.

Yildirim et al. (2002b) suggested that pigmentation, lactose fermentation, serotype, growth properties in fluid medium and in soft agar may be generally used to distinguish between *S. agalactiae* isolates from cattle, humans, dogs, and cats. However, the results were not reported from all of cattle isolates. The results also showed that *S. agalactiae* isolates from dogs, cats, and humans were generally pigmented, lactose negative, and did not hemagglutinate rabbit erythrocytes. In contrast, they reported that cattle *S. agalactiae* isolates were nonpigmented, utilized lactose, hemagglutinated rabbit erythrocytes, were generally non-typable with a few IV and Ia serotypes, and had sedimentary growth properties in fluid medium and diffuse colony morphology in soft agar. Like Yildirim et al. (2002b), we observed that *S. agalactiae* isolates from most of the dairy cattle with mastitis in the present study were nonpigmented, always

fermented lactose, were non-typable, and exhibited sedimentary growth patterns in fluid medium. Merl et al. (2003) observed that cattle isolates were generally non-typable, expressed sedimentary growth in fluid medium and diffuse colony morphology on soft agar, but were pigmented. The only bacteriological characteristic difference between their study and those of the present study was pigmentation.

S. agalactiae is the cause of severe disease affecting both fish and cattle. However, the possibility of disease transmission between cattle and fish has not been established. The results of the present study indicate that cattle isolates are not experimentally infectious for Nile tilapia and channel catfish in the laboratory. The results indicate that cattle isolates are not likely a source of infection in fish. The reasons for the lack of virulence of the cattle isolates for tilapia and catfish are unknown. It appears that the cattle isolates failed to survive in the fish hosts. The transmission of *S. agalactiae* within fish species and between fish species is the most likely source of *S. agalactiae* infection in fish (Evans et al., 2002). Evans et al. (2002) demonstrated that Nile tilapia was susceptible to mullet and seabream isolates of *S. agalactiae*.

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